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pyewacket, a new zebrafish fin pigment pattern mutant

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Summary

Many mutants that disrupt zebrafish embryonic pigment pattern have been isolated, and subsequent cloning of the mutated genes causing these phenotypes has contributed to our understanding of pigment cell development. However, few mutants have been identified that specifically affect development of the adult pigment pattern. Through a mutant screen for adult pigment pattern phenotypes, we identified *pyewacket* (*pye*), a novel zebrafish mutant in which development of the adult caudal fin pigment pattern is aberrant. Specifically, *pye* mutants have fin melanocyte pigment pattern defects and fewer xanthophores than wild-type fins. We mapped *pye* to an interval where a single gene, the zebrafish ortholog of the human gene DHRSX, is present. *pye* will be an informative mutant for understanding how xanthophores and melanocytes interact to form the pigment pattern of the adult zebrafish fin.

Key words: *pyewacket*/zebrafish/pigment pattern/melanocytes/xanthophores

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Introduction

While identification of numerous mutations affecting zebrafish embryonic development has led to subsequent cloning of many genes required for embryonic organ and tissue patterning, less is known about the genes and pathways required for formation of adult zebrafish structures. Yet identifying mechanisms required for formation of adult structures is equally important, as it will lead to a better understanding of how human adult onset diseases develop. Many poikilotherms, including amphibians, anurans and fish, undergo metamorphosis to become adults, making these systems advantageous tools for dissecting requirements for adult tissue development.

One model system used to study pathways and mechanisms required for adult pattern formation is the zebrafish pigment pattern. The pigment pattern of zebrafish is complex, and consists of several different types of pigment cells including black melanocytes, yellow xanthophores and iridescent iridophores. The larval pigment pattern of the fish is established by day 3 and includes melanocytes arranged in two dorsal stripes, melanocytes in stripes within the horizontal myoseptum along the lateral line, and two ventral stripes of melanocytes (reviewed in Quigley and Parichy, 2002). Xanthophores are found over the head and along the dorsum of the larvae and iridophores are on the fin folds and interspersed with dorsal melanocytes. During pigment pattern metamorphosis, this larval pattern is replaced by the adult pattern of four horizontal stripes along either side of the fish containing iridophores, xanthophores and melanocytes, and interstripe regions containing only xanthophores and iridophores (Hirata et al., 2003; Johnson et al., 1995a; Parichy et al., 2003). The adult zebrafish caudal fin also has three horizontal melanocyte and xanthophore stripes alternating with interstripe regions containing only xanthophores (Hirata et al., 2005). This creates the characteristic alternating black and yellow stripes in the fish.

Many mutants that affect both embryonic pigment pattern and adult pigment pattern have been identified including *nacre (mitf), sparse (kit)*, and *panther (csf1r)*; Lister et al., 1999; Parichy et al., 1999, 2000a). In addition to this class of mutants, there are some mutations that specifically affect adult pigment pattern, including *leopard*, *rose* (*ednrB*) and *puma* (Johnson et al., 1995a; Parichy et al., 2000b, 2003), and some that only affect embryonic zebrafish pigment pattern such as *shady* (Kelsh et al., 1996). All of these classes of mutations suggest that there are differences between larval pigment cells and pigment cells in the adult fish. Whether these differences exist because of different pigment cell precursor populations or differences in mechanisms of development or a combination of the two has yet to be determined.

Mutations affecting adult melanocytes have revealed two zebrafish melanocyte mutant epistasis groups that in turn identify different populations of melanocytes

within the adult zebrafish body and fin pigment pattern. Mutants for *panther* (*csf1r*), *rose* (*ednrB*), and *leopard* all have approximately half the number of body stripe melanocytes found in wild-type zebrafish (Johnson et al., 1995a; Parichy et al., 2000a,b). *kit* mutants also have about half the number body melanocytes found in wild-type zebrafish (Parichy et al., 1999). Double mutants for *kit* and any one of the mutants in the first group lack almost all of their body melanocytes, indicating that there are two different melanocyte types in the adult zebrafish body stripes, *kit*-dependent melanocytes, and *csf1r*-, *ednrB*-, or *leopard*-dependent melanocytes (Johnson et al., 1995a).

Another population of melanocytes is found in zebrafish fins. These melanocytes are revealed in *kit* single mutants, where they differentiate in response to the absence of primary melanocytes that differentiate in wild-type fins at early stages of ontogeny or regeneration (Rawls and Johnson, 2000). Double mutants for *kit* and any mutant in the second epistasis group retain these melanocytes in their fins as well (Johnson et al., 1995a; Parichy et al., 2000a). Thus, these regulatory fin melanocytes are genetically and temporally distinct from the vast majority of fin melanocytes that develop in wild-type or *csf1r*, *ednrb* and *leopard* single mutants. Therefore, in addition to the two populations of melanocytes in the zebrafish body that fail to complement for deficiency, there are two populations of melanocytes in zebrafish fins, one of which is genetically separable from adult body melanocytes.

While all of these mutants have revealed different populations of pigment cells in the adult zebrafish and some of the genes required for their development, we still know relatively little about how these cells interact to form the adult pigment pattern. A few of these mutants, specifically *panther*, *puma* and *leopard*, have pigment cell patterning defects in addition to lacking specific pigment cell types. The identification of *panther* as the *csf1r* receptor tyrosine kinase has given us some insight into the genetic control of these processes, and molecular identification of *puma* and *leopard* will also inform us of the genetic control of these processes. Analysis of *csf1r* mutants revealed the importance of xanthophores in patterning melanocytes into stripes, indicating that communication between pigment cell types is crucial for proper pigment pattern formation (Goodrich and Greene, 1959; Goodrich et al., 1954; Parichy and Turner, 2003). We undertook a screen to identify new mutants involved in adult pigment pattern formation with the hopes of identifying new pathways involved in pigment pattern formation. Here we report on a new adult pigment pattern mutant, *pyewacket* (*pye*) that specifically affects the fin pigment pattern. We find that *pye* mutants have a wild-type like embryonic pigment pattern, but as adults they have fewer caudal fin xanthophores. *pye* mutants also have fin melanocyte patterning defects. Identifying the molecular nature of *pye* and how *pye* is required for xanthophore development and melanocyte patterning will give us more insight into the genetic control of adult pigment pattern development and how the interaction of pigment cells is important for this process.

Results and discussion

Identification of *pyewacket* mutant and phenotypic characterization

To identify mutations affecting development of the adult zebrafish pigment pattern, we screened for mutant zebrafish with defects in adult pigment stripe formation. ENU mutagenized males were crossed to females to generate mothers heterozygous for induced mutations. A large number of our crosses were either homozygous or heterozygous for the *sparse*(*kit*) mutation, which would enable us to identify mutations that disrupt pigment pattern in *kit*-independent melanocytes as well as *kit*-dependent melanocytes in the wild-type background. We then performed early pressure parthenogenesis (EP; Johnson et al., 1995b; Streisinger et al., 1981) to generate homozygous mutant progeny from these mothers. By screening progeny from 952 mothers with presumptive heterozygous mutations, we identified one clutch where melanocyte development was perturbed in half the fish. These fish were also mutant for *kit*, and while body melanocyte stripes appeared normal, the caudal and anal fin melanocytes were not arranged in the characteristic stripes. Instead, melanocytes were dispersed across the proximal part of the anal and caudal fins (Figure 1A, C), raising the possibility that this mutation affected the development of the *kit*-independent melanocyte population. In addition, while we did not observe an iridophore deficit, these double mutants had less yellow pigmentation than *kit* mutants by themselves in the body and fins, indicating there may be a defect in xanthophores. When we isolated the homozygous mutation away from *kit*, we found that these fish had an independent pigment pattern phenotype as well (Figure 1B, D–F). In these mutants, caudal and anal fin melanocytes remained more dispersed than in wild type. As in the double mutants, we observed no deficit in iridophores but did observe less of the yellow xanthophore pigment in the single mutant background in the body and fins. In contrast to these adult pigment pattern defects, we observed no defect in embryo or larvae pigment pattern, as these mutants appear to have the same number and pattern of melanocytes and the same intensity and localization of the yellow pigment of the xanthophores. We decided to call this new mutant that specifically affects adult xanthophores and disrupts the pigment pattern of the adult fins *pyewacket* (*pye*).

We next examined the *pye* mutant for melanocyte and xanthophore phenotypes during development, focusing on the caudal fin. In addition to the *pye* pigment pattern defects, we observe that *pye* fish may

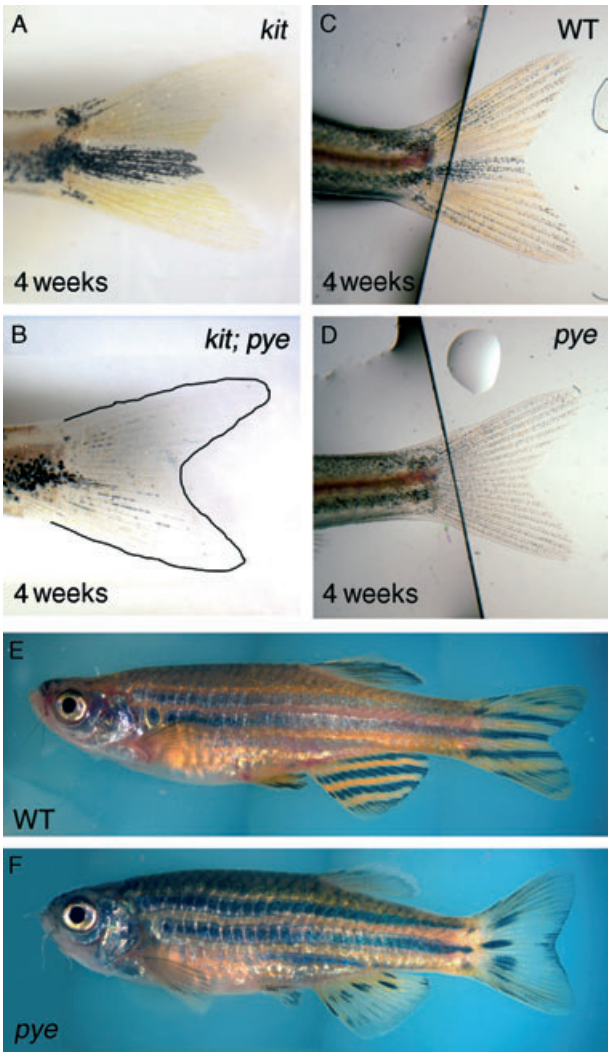

Figure 1. *pyewacket* fins have fewer xanthophores and disrupted melanocyte patterning. The *pye* mutant was originally isolated as a *kit; pye* double mutant (C), where melanocytes are not organized into stripes as they are in *kit* single mutants (A). Note the reduced yellow (xanthophore) pigment in the outlined fin in (C). *pye* single mutant fins (D) have melanocytes dispersed throughout the fin and fewer xanthophores while wild-type fins (B) have distinct melanocyte and xanthophore stripes. Whole body images of adult wild-type (E) and *pyewacket* (F) male fish. Differences in pigment pattern are restricted to the caudal and anal fins. Difference in appearance of body melanocyte stripes between these two fish results from additional contraction of melanosomes in the wild-type individual, because of slight differences in handling the fish for photography, rather than effects of *pyewacket* on body stripe pigmentation.

have a slight general growth defect, as within a population of *pye* and wild-type siblings, *pye* mutants appeared smaller than their wild-type siblings overall. Thus, in our experiments, we selected *pye* and wild-type siblings that were similar in size. We found that in both *pye* and wild-type fins at 3 weeks of age, melanocytes are initially dispersed throughout the caudal fin (Figure 2A, B). However, at 3 weeks, we also already see a difference in the number of visible xanthophores between wild-type and *pye* fins, with wild-type fins having an average of 196 ± 10 xanthophores per fin and *pye* mutants having an average of 77 ± 28 xanthophores per fin. At 4–5 weeks, wild-type fin melanocytes have begun organizing into stripes, and xanthophores still appear in the two outer stripes but now appear to be excluded from the inner stripe (Figure 2C, D). At this stage in *pye* mutant fins, melanocytes fail to organize into stripes and are still dispersed throughout the caudal fin. Additionally, the few xanthophores that are present are distributed throughout the fin interspersed with the melanocytes. Finally, at 7 weeks, wild-type fins have pigmented xanthophores and melanocytes organized into alternating stripes. At this stage in *pye* mutants, melanocytes and xanthophores are organized in distinct patches in the caudal fin but are not organized into stripes (Figure 2E, F). As recent TEM studies have revealed there are faintly pigmented xanthophores in the stripe region of wild-type zebrafish fins underneath the melanocytes (Hirata et al., 2005), our counts of xanthophores may miss those xanthophores that are obscured by melanocytes. Thus, the difference in wild-type and *pye* xanthophore numbers, we observe only applies to the xanthophores that are not obscured by melanocytes.

Our finding that *pye* mutant fins appear to have fewer pigmented xanthophores than wild-type fins led us to examine the number of melanocytes in *pye* fins. If xanthophores and melanocytes share a common precursor, it could be that in *pye* mutants, more progeny of this common precursor differentiate into melanocytes than xanthophores. However, at 4 weeks, we found the total number of melanocytes in caudal fins is similar between wild type (average of 777 ± 98) and *pye* (average of 770 ± 62), indicating that the *pye* phenotype is not likely to be the result of aberrant cell fate allocation of a common melanocyte and xanthophore precursor.

We also used fin regeneration to examine the development of the *pye* phenotype. During regeneration, wild-type fins and *pye* mutant fins appeared similar at first, with melanocytes appearing dispersed throughout the regenerate at 4–5 days (not shown). However, as the regenerates grew, the xanthophore containing region expanded distally in wild-type fins and failed to expand distally in *pye* mutant regenerating fins (not shown). Counts of pigment cells in wild-type and *pye* regenerating fins revealed a similar phenotype to that observed for ontogenic fin growth with similar melanocyte numbers to wild-type fins (440 ± 92 in wild type and 404 ± 88 in *pye* in day 7 regenerates) and fewer xanthophores (Figure 2G, H; 149 ± 68 in wild type and 45 ± 12 in *pye* in day 7 regenerates) in *pye* regenerating fins. In addition to the pigment pattern defect, we also observed that *pye* mutant fins regenerated more slowly than wild-type fins, indicating that *pye* may act on

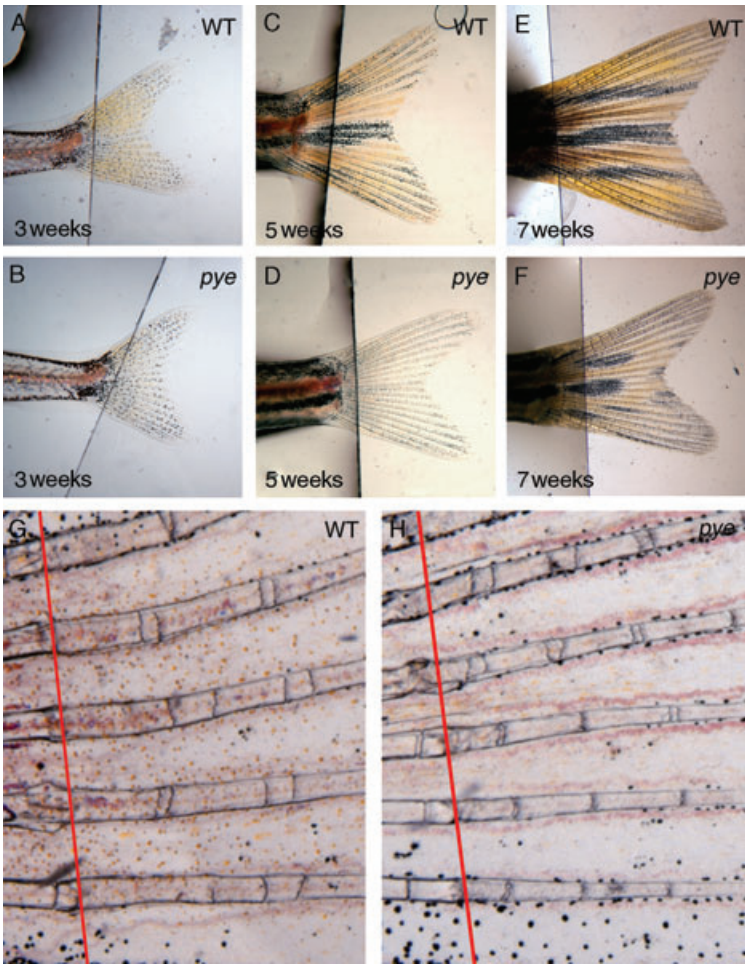

Figure 2. Ontogeny of *pyewacket* fins (A–F). At 3 weeks, WT (A) fins have melanocytes dispersed throughout the caudal fin and xanthophores dispersed throughout the fin as well. *pye* (B) fins also have melanocytes dispersed throughout the fin but have fewer xanthophores than WT. At 5 weeks, WT fins (C) have formed a central melanocyte stripe surrounded by xanthophores, while *pye* (D) fins continue to have fewer xanthophores and melanocytes are still dispersed throughout the fin. 7 week WT fins (E) have three distinct melanocyte stripes and xanthophore stripes alternating with melanocyte stripes, while *pye* fins (F) have patches of melanocytes and some xanthophores dispersed throughout the fin. *pye* day 9 regenerating fins have fewer xanthophores than wild-type regenerating fins (G–H). Before these images were taken, fish were immersed in epinephrine for 5 min to contract melanosomes. Both images show the same five caudal fin rays of the dorsal xanthophore stripe region. Anterior is to the right in both images and red lines indicate the amputation plane.

tissues other than the pigmentary system. Because of this defect in regeneration, for melanocyte and xanthophore counts we compared similar stages of wild-type and *pye* fins by counting pigment cells in fins that had regenerated the same number of caudal fin segments.

We find that *pye* mutants have fewer xanthophores in their caudal fins and fin melanocytes fail to organize into stripes. A similar defect is observed in *csf1r*(*panther*) mutants, where xanthophores fail to develop and melanocyte patterning is abnormal as a consequence of the xanthophore defects (Parichy and Turner, 2003; Parichy et al., 2000a). *pye*, like *csf1r*, could be required directly for xanthophore development, and indirectly for melanocyte patterning. *pye* could be required in xanthophores for the correct number of xanthophore precursors, either through survival of these precursors or through division of these precursors. Alternatively, *pye* could be required for migration of xanthophore precursors into the developing fin or proper differentiation of xanthophores. *pye* could promote one or a combination of these processes by acting within the xanthophores themselves or, alternatively, could contribute to an external signal that xanthophores must recognize for proper development. Future studies with expression of xanthophore precursor markers in the developing fin could allow for distinction between these different possibilities.

An alternative to the notion that *pye* acts on xanthophores which in turn pattern melanocytes is that *pye* affects melanocytes and xanthophores independently. If *pye* is required in melanocytes independently of the xanthophore requirement, it could be an intrinsic factor required for melanocytes to recognize their environment and migrate or die to form stripes, or part of an extrinsic signal guiding these processes. Regardless of how *pye* is required for melanocyte patterning, such a requirement might be specific for zebrafish fin melanocytes, as body melanocytes are able to organize into stripes in *pye* mutants (Figure 1E, F). One intriguing possibility is that *pye* is required for development of all adult xanthophores, but specifically acts on caudal fin melanocytes to promote their ability to recognize signals from xanthophores to form stripes. This is a plausible model because we know from previous studies that there is a genetically distinct population of melanocytes, termed regulatory melanocytes, in zebrafish fins that is both *kit*- and *csf1r*-independent for development (Johnson et al., 1995a; Rawls and Johnson, 2000). Our data is consis-

tent with the idea that these regulatory melanocytes may require *pye* for interaction with xanthophores.

One difference between *pye* and the *csf1r* mutant phenotype is that *csf1r* mutants also have defects in body melanocyte stripe patterning (Parichy et al., 2000a,b). This difference could suggest that there are different populations of xanthophores identified by their response to mutations just as different populations of melanocytes in the body and fin of zebrafish are identified by their deficits in different mutants. While *csf1r* appears to be required for proper differentiation and migration of all xanthophores, *pye* could be required only for a subset of xanthophores. There are a few xanthophores present in *pye* mutant fins and, although there appear to be fewer xanthophores in the body as well, the melanocyte body stripes of *pye* mutants develop properly. One possibility is that different populations of xanthophores have roles of patterning melanocytes in the body and in the fin, and *pye* specifically affects the fin population of xanthophores.

Another possibility for why the *pye* mutant has defects in fin melanocyte patterning but not body melanocyte patterning is that the allele of *pye* we isolated, pye^{j20e1}, is not a null mutation. If the pye^{j20e1} mutation is hypomorphic, it could be that a null allele of *pye* would resemble the *csf1r* phenotype, and *pye* is actually required in all xanthophores for their development. It is also possible that the difference between *pye* and *csf1r* phenotypes could reflect different sensitivities of melanocytes to the absence of xanthophores. The *pye* phenotype could suggest that melanocytes in the caudal fin are more sensitive to the absence of xanthophores than are melanocytes in the body stripes. Whichever of these ideas is correct, *pye* remains an intriguing mutant for identifying how pigment pattern of the adult zebrafish is established.

Genomic location of *pye* mutation

Because the proportion of *pye* mutants in the founding EP clutch suggested tight centromere linkage, we initially mapped the *pye* mutant to chromosome 1 using a collection of first pass centromere-linked markers (Rawls et al., 2003). Once we determined *pye* was near the centromere of chromosome 1, we identified close markers on either side of the mutation and used these to screen 1623 mapping cross progeny for individuals with a recombination event between these close markers. We identified 11 recombinant individuals between the flanking markers z21260 and z1351. To further refine the map, we developed new markers and used these to more precisely delimit the region for *pyewacket*. This allowed us to define a 140-kb critical region containing the *pye* mutation (Figure 3), between the markers 99332 and 106G5-109. Our analysis shows that markers to the right of the *pye* mutation are in the region 29.082–29.260 Mb on chromosome 1 (Sanger Institute zebrafish genome assembly version 5), while markers to the left of *pye* are on the region from 29.875 to 31.148 Mb. In addition, our analysis suggests that these two regions of chromosome 1 assembly sequence are bridged by sequence from the chromosome 19 assembly (12.395–12.438 Mb) and sequence from Zv5 scaffold NA14443 (66.000–67.550 Kb). We find that sequence from one end of the chromosome 19 assembly sequence overlaps the left chromosome 1 segment on one side of *pyewacket*, and that one end of scaffold sequence from NA14443 overlaps the chromosome 1 region to the right of *pyewacket*. While we find no overlap between the chromosome 19 and NA14443 sequence, RT PCR analysis reveals that we can amplify from transcripts bridging these assembly fragments, confirming our juxtaposition of these sequences (not shown). While our analysis differs from the current Sanger Institute assembly of this region, this assembly is a work in progress, meaning that there may be misassembled regions such as this that have not yet been corrected.

We find only a single gene in the *pye* candidate region between markers 99332 and 106G5-109, the zebrafish ortholog of the human DHRSX gene. Two zebrafish ESTs (Genbank accession numbers CK126256

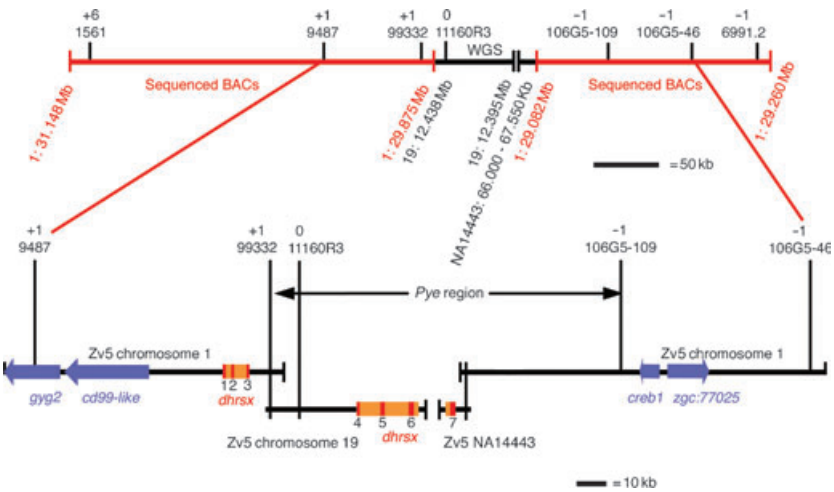

Figure 3. Our interpretation of the chromosomal region surrounding *pye* is shown in red, indicating regions on chromosome 1 covered by sequenced BACs, and black, indicating the chromosome 19 and NA14443 regions covered by whole genome shotgun sequence (WGS). Below this is a blow up of the *pye* critical region, showing the region between the markers 9487 and 106G5-46. The coding sequence of the *dhrsx* exons are numbered and shown in red. A marker that is one recombination event to the left of *pye* (99332) is in intron 3 of *dhrsx*, while 106G5-109, one recombination event to the right, is distal to exon 7 of *dhrsx*.

and CN839995) in the *pyewacket* candidate region are part of the 3′UTR of this gene. We identified exons 1–3 of the zebrafish *dhrsx* gene on chromosome 1 sequence between 29.887523 and 29.895301 Mb and exons 4–6 on chromosome 19 between 12.395298 and 12.414788 Mb. We find exon 7 on NA14443 between 66.000 and 67.550 Kb. We find one recombination event within intron 3 (a large 35–40 kb intron) of this gene. Because recombination indicates that the *pye* mutation is to the right of exon 3, we sequenced exons 4–6 and exon 7 coding sequence of the *dhrsx* gene. Sequencing the *pye* coding sequence of exons 4–7 in *pye* and the unmutagenized wild-type background revealed no coding sequence changes. There is unlikely to be in another gene within the *pye* critical region, as BLAST searches to protein databases and EST databases fail to identify other potential genes or transcripts in this critical region. Confirming that this gene is expressed in zebrafish, we were able to amplify the *dhrsx* gene from cDNA of 20 hpf embryos, growing fins, and d3 and d6 regenerating fins. Amplification of the 3′UTR of *dhrsx* by 3′race identifies *dhrsx* has a 3′UTR approximately 2 kb in length. *In situ* hybridizations with a probe recognizing 750 bases of *dhrsx* showed ubiquitous expression in 20 hpf embryos (not shown). We also observed faint, possibly ubiquitous expression in fins. In light of the fact that we failed to identify a coding sequence change in *dhrsx* between *pye* mutants and the founding background, we assayed expression of *dhrsx* in *pye* and wild-type regenerating and non-regenerating fins by quantitative PCR. We found no difference in the levels of *dhrsx* expression between *pye* and wild-type fins (not shown).

The *dhrsx* gene coding sequence suggests this gene is a member of the short-chain dehydrogenase/reductase (SDR) gene family. The predicted protein sequence of this gene contains the characteristic NAD or NADP binding domain sequence GXXXGXG (GGTRGMG) and the substrate-binding domain YXXXK (YAQSK). The human DHRSX gene is expressed in several tissues including the heart, skeletal muscle, pancreas, kidney and thymus (Gianfrancesco et al., 2001). However, the substrate and function of this protein are unknown. Human DHRSX is located on the pseudoautosomal region of the X/Y chromosome, and several genes in this region on the human chromosome are also present in the zebrafish *dhrsx* region, including zebrafish *cd99-like*, *gyg2, adlican* and *prkx* (Figure 3 and not shown). This indicates that this region of the zebrafish genome has synteny with part of the psuedoautosomal region of the X/Y chromosome, confirming the orthology suggested by similarity of human and zebrafish *dhrsx*.

Our mapping of the *pye* mutation to chromosome 1 reveals only *dhrsx* as a candidate in the *pye* critical region. Sequence analysis revealed no coding sequence changes and quantitative PCR revealed no changes in transcript level. Thus, if *dhrsx* is the responsible gene, it may be the result of an unidentified lesion in the transcript that prevents proper expression of the protein. Alternatively, *pye* may be a mutation in a regulatory element that changes spatial or temporal expression of *dhrsx* without grossly changing transcript levels as assessed by Q-PCR of the gene. An alternative to the notion that the mutation is in *dhrsx* is that it affects some other gene not recognizable by BLAST analysis (such as non-coding RNAs).

The substrate of the *dhrsx* gene is not defined, but sequence similarity suggests that it is closest to the retinol dehydrogenase family of SDR short-chain dehydrogenases, thus it may have a similar role in retinoic acid (RA) metabolism. One idea of an alternative role for *dhrsx* other than being involved in retinoic acid signaling comes from other SDR family members (such as sepiapterin reductase; Negishi et al., 2003), which are involved in xanthophore sepiapterin pigment synthesis, raising the possibility that *dhrsx* may be required in a similar fashion, or to convert a pigment by-product to a signaling molecule. If *dhrsx* is the mutated gene in *pye*, identifying the substrate of *dhrsx* and other members of the pathway will identify a novel mechanism involved in pigment cell differentiation and pigment pattern formation.

We have identified a zebrafish pigment pattern mutant that has fin specific pigment pattern defects. The paucity of xanthophores and the disrupted pattern of melanocytes in *pye* fins suggests that the defect in this mutant affects the interactions of these two pigment cell types. Thus, no matter what the molecular nature of the *pye* mutation is, this mutant will be an informative tool for exploring how pigment cells interact to promote proper pattern formation.

Materials and methods

Stocks and screen for new mutations

To screen for mutations in adult pigment pattern development, random mutations were generated by soaking male zebrafish of the C32 strain in *N*-ethyl-*N*-nitrosourea for 15–20 min and repeating this process 3–4 times (Solnica-Krezel et al., 1994). These males were then crossed to C32 females to generate fish with presumptive heterozygous mutations. Some of the mutagenized males were also *sparse*(*kit*) mutants, as were some of the mothers, meaning that the fish that were heterozygous for new mutations from this cross could be homozygous or heterozygous for the *kit* mutation as well. Eggs from these female fish presumptively heterozygous for new mutations were subjected to early pressure (EP) parthenogenesis (Johnson et al., 1995b; Streisinger et al., 1981) to generated half tetrad diploids, and the progeny were screened at 4 weeks for mutant phenotypes. Following the lab's tradition of using cat names for pigment pattern mutants, we decided to call the new adult pigment pattern mutant we identified *pyewacket* (*pye*), after the pet siamese familiar of Kim Novak in the 1958 movie *Bell, Book and Candle*.

Generation of *pyewacket* genomic map

After identification of the *pyewacket* mutant phenotype, *pye* homozygotes were outcrossed to individuals of the SJD mapping strain.

These *pye*/SJD heterozygotes were backcrossed to *pye* homozygous fish to generate mapping cross individuals for bulk segregate mapping and subsequent mapping by recombination (Rawls et al., 2003). Recombinant individuals were first identified using available zebrafish genomic markers (Shimoda et al., 1999), and the recombination sites in these individuals were further refined using new polymorphic markers developed using the Sanger Institute's Whole Genome Shotgun Assembly sequence or from Washington University zebrafish EST assemblies (http://zfish.wustl.edu) Size polymorphisms were visualized by running PCR reactions on 1.5% agarose gels. The 5′–3′ primer sequences for these size polymorphism markers are as follows (F = forward primer, R = reverse primer): 1561 F- TGTTAGTGCAGTATTTGTATTGATGG, R-CCTGATGAATAAAGGGACTAAGC; 9487 F-TTTTATGCTGCTGCAAATCC, R-GGATCATACATTGTCTTTAAATTCAGC; 11160R3 F-TCAAACAAATCTCCCTTTGG, R-TTGTCCTGATTTACATGCATCC; 106G5-109 F-CATCATTTGTTACGAGTTCTTTGC, R-TCGTGTCAAGTCCTTTGGAA; 106G5-46 F-GCCTATGTTAGATTGCAGTGTCTG, R-GCTTTGACCACCTGTTCGTT; 6991.2 F-GATGCACTCCGCTTACACG, R-GGACGGAGAGCAATTGAGG. SNP marker 99332 was identified from sequencing reactions performed with Amersham Sequenase and an ABI 3100 sequencer. The 5′–3′ primer sequences for 99332 were F-TTGTGCCCCATATCACAGC and R-CGGCGATTCTCTCAGATAGC, and the sequence polymorphism detected was at position 133 of the 414 bp amplified sequence (*pye* = AACGTA, SJD = AATGTA).

Imaging and pigment cell counts

Images were taken with a Jenoptik ProgRes C14 camera (Jenoptik AG, Jena, Germany) mounted onto a Nikon SMZ1500 microscope (Nikon Corporation, Tokyo, Japan) or an Olympus SZX12 microscope (Olympus Corporation, Tokyo, Japan). The fish were treated with tricaine for 2–5 min and then placed onto slides with their caudal fins under glass coverslips for better imaging. The resulting images were used for melanocyte and xanthophore counts after transferring to Adobe Photoshop.

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